FEBS Letters 581 (2007) 453–461

Nitrosative stress in plants

Raquel Valderrama[a], Francisco J. Corpas[b,*], Alfonso Carreras[a], Ana Fernández-Ocaña[a], Mounira Chaki[a], Francisco Luque[a], María V. Gómez-Rodríguez[a], Pilar Colmenero-Varea[a], Luis A. del Río[b], Juan B. Barroso[a]

[a] *Grupo de Señalización Molecular y Sistemas Antioxidantes en Plantas, Unidad Asociada al CSIC (EEZ), Área de Bioquímica y Biología Molecular, Universidad de Jaén, E-23071 Jaén, Spain*
[b] *Departamento de Bioquímica, Biología Celular y Molecular de Plantas, Estación Experimental del Zaidín (EEZ), CSIC, Apartado 419, E-18080 Granada, Spain*

Received 22 November 2006; revised 24 December 2006; accepted 5 January 2007

Available online 16 January 2007

Edited by Mark Stitt

Abstract **Nitrosative stress has become a usual term in the physiology of nitric oxide in mammalian systems. However, in plants there is much less information on this type of stress. Using olive leaves as experimental model, the effect of salinity on the potential induction of nitrosative stress was studied. The enzymatic L-arginine-dependent production of nitric oxide (NOS activity) was measured by ozone chemiluminiscence. The specific activity of NOS in olive leaves was 0.280 nmol NO mg^{-1} protein min^{-1}, and was dependent on L-arginine, NADPH and calcium. Salt stress (200 mM NaCl) caused an increase of the L-arginine-dependent production of nitric oxide (NO), total *S*-nitrosothiols (RSNO) and number of proteins that underwent tyrosine nitration. Confocal laser scanning microscopy analysis using either specific fluorescent probes for NO and RSNO or antibodies to *S*-nitrosoglutathione and 3-nitrotyrosine, showed also a general increase of these reactive nitrogen species (RNS) mainly in the vascular tissue. Taken together, these findings show that in olive leaves salinity induces nitrosative stress, and vascular tissues could play an important role in the redistribution of NO-derived molecules during nitrosative stress.**

Keywords: Nitric oxide; Nitric oxide synthase; *S*-Nitrosothiols; *S*-Nitrosoglutathione; Nitrosative stress; Nitrosylation; Nitrotyrosine; Peroxynitrite; Olive; salinity; *Olea europaea* L.

1. Introduction

Parallel to the expression reactive oxygen species (ROS), the term "reactive nitrogen species" (RNS) was coined to designate nitric oxide (NO) and a family of related molecules such as peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3), *S*-nitrosoglutathione (GSNO), nitrogen dioxide ($NO_2^{\cdot}$), nitrosyl cation (NO^+), etc. [1,2]. In an analogous manner to the term oxidative stress, recently in animal systems the expression nitrosative stress started to be employed to define the de-regulated synthesis or overproduction of NO and NO-derived products and its toxic physiological consequences [3].

In plant cells there is increasing evidence that nitric oxide (NO) has an important function in plant growth and development and is also a key signalling molecule in different intracellular processes [4–10]. However, although intensive work is being done on the physiological function of NO under normal and stress conditions, much less information is available on other RNS, like *S*-nitrosoglutathione, peroxynitrite, and nitrotyrosine.

Salinity by NaCl is one of the major abiotic stresses affecting plant productivity due to the alterations produced in photosynthesis and respiration, and in the metabolism of proteins and nucleic acids [11]. Recent studies carried out in our laboratory on the effect of salt stress in olive plants showed that salinity produced in leaves an imbalance between the ROS production and antioxidant defences, with the induction of oxidative stress [12]. However, in plants there is very little information on the involvement of nitrosative stress in the toxicity produced by adverse abiotic conditions.

In this work, using olive plants evidence is presented showing that under NaCl stress, NO and other NO-derived products are overproduced, indicating that in plant cells nitrosative stress could participate, as a significant component, in the mechanism of damage produced by abiotic toxic conditions.

2. Materials and methods

2.1. Plant material and in vitro culture conditions

Olive (*Olea europaea* L., cv. Manzanillo) seeds were provided by the *Dpto. de Olivicultura y Arboricultura Frutal* (CIDA, Córdoba, Spain) and were germinated under in vitro conditions. Seedlings were grown in the dark at 13 °C for 15 d in an embryos medium and then were transferred to a DKW medium. These cultures were grown in a temperature-controlled chamber at 25 °C for another 30 d, with a 16 h photoperiod under Sylvania Gro-Lux lighting with a photon flux density of 130–140 μmol m^{-2} s^{-1}. For the experiments with NaCl, seedlings were transferred to nutrient media with NaCl 200 mM. They were kept during 21 d at the same growth conditions, and then were used for the analyses [12].

*Corresponding author.
E-mail address: javier.corpas@eez.csic.es (F.J. Corpas).

Abbreviations: AG, aminoguanidine; CLSM, confocal laser scanning microscopy; DAF-2 DA, 4,5-diaminofluorescein diacetate; DCF-DA, 2′,7′-dichlorofluorescein diacetate; DHE, dihydroethidium; GSNO, *S*-nitrosoglutathione; NO, nitric oxide; NOS, nitric oxide synthase; *n*-Tyr, 3-nitrotyrosine; PTIO, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl; ROS, reactive oxygen species; RNS, reactive nitrogen species; RSNO, *S*-nitrosothiols; TMP, 2,2,6,6-tetramethyl-piperidine

0014-5793/$32.00
doi:10.1016/j.febslet.2007.01.006

2.2. Crude extracts of olive leaves

All operations were performed at 0–4 °C. Leaves (1 g FW) were ground to a powder in a mortar with liquid nitrogen, and were suspended in 3 ml of 100 mM Tris–HCl buffer, pH 8.0, containing 1 mM EDTA, 1 mM EGTA, 0.1 M NaCl, 7 % (W/V) PVPP, 15 mM DTT, 10 μg/μl calmodulin, 10 μM BH_4, 15 mM PMSF and a commercial cocktail of protease inhibitors (AEBSF, 1,10-phenantroline, pepstatin A, leupeptine, bestatine and E-64 from Sigma) (1:3; FW/V). Homogenates were filtered through one layer of miracloth (Calbiochem) and centrifuged at $3000 \times g$ for 5 min. Then, the supernatants were passed through NAP-10 columns (Amersham-Biosciences). For Western-blots, the columns were equilibrated with 10 mM Na-phosphate buffer, pH 6.8, and eluted with 10 mM K-phosphate buffer, pH 7.8. The average concentration of proteins in leaf extracts from control and NaCl-treated olive plants was 0.57 and 0.49 μg μl^{-1}, respectively.

2.3. Quantification of NO production from L-arginine (NOS activity)

The production of NO from L-arginine was estimated by ozone-chemiluminescence using a Nitric Oxide Analyser (NOA 280i, Sievers Instruments, Boulder, CO, USA). To avoid any potential inferences by endogenous nitrite/nitrate the samples were passed through Sephadex G-25 desalting columns (NP-10 from Amersham) which eliminated low molecular weight substances ($M_r < 1000$). The columns were equilibrated and eluted with 40 mM HEPES buffer, pH 7.2, 0.2 mM Chaps, 15 mM DTT, 1.25 mM $CaCl_2$, 1 mM β-NADPH, 5 μg/ml calmodulin, 10 μM FAD, 10 μM FMN, 10 μM BH_4. After that the NOS activity was performed in duplicate, for each sample, in a reaction medium containing 40 mM HEPES buffer, pH 7.2, 0.2 mM Chaps, 10 μg/ml calmodulin, 1.25 mM $CaCl_2$, 1 mM β-NADPH, 10 μM FAD, 10 μM FMN, 10 μM BH_4, and 1 mM L-Arg. The reaction mixture was incubated at 37 °C for 30 min. The NO produced in the enzymatic reaction is quickly oxidized to its stable end products nitrite and nitrate. At 0 time and 30 min, 200 μl of the reaction medium was deproteinated by the addition of 100 μl of 0.8 N NaOH and 100 μl of 16% $ZnSO_4$, and the mixture was shaken vigorously for 30 s and centrifuged for 10 min at $16000 \times g$. Forty microliter aliquots of supernatants were injected into the purge chamber of the nitric oxide analyzer (NOA) containing 5 ml of 50 mM vanadium trichloride (VCl_3) in 1 M HCl at 90 °C under an atmosphere of nitrogen (N_2). In these conditions, the NO-derived nitrite/nitrate of samples is reduced back to NO which was mixed with ozone, generated in the NOA, and the light emitted from the chemiluminiscence reaction between NO and ozone was detected by a photomultipler tube. The amount of NO generated was computed by referring the magnitude of these signals to that of those generated by nitrate standards. The production of NO in the 30 min of reaction was calculated by subtracting the blank value (zero time), which represented the non-enzymatic NO production, and the activity was expressed as nmol of NO mg^{-1} protein min^{-1}. As negative controls, samples were preheated at 95 °C for 10 min. To validate this method to determine the NOS activity of plant samples, commercial rat neuronal NOS from Calbiochem (2.9 U) was assayed separately as positive control. The NOS activity was also determined using a concentration of 100 μM L-Arg and in the presence of 1 mM aminoguanidine which is an irreversible inhibitor of both constitutive and inducible NOS activity in animal cells [13].

2.4. Chemiluminescence detection of total S-nitrosothiols (RSNOs)

Total RSNOs were estimated by a chemiluminescence method, as described by Jourd'Heuil et al. [14] with some modifications. The detection of RSNOs is based on the reductive decomposition of nitroso species by an iodine/triiodide mixture to release NO, which is subsequently measured by gas-phase chemiluminescence upon reaction with ozone. RSNOs are sensitive to mercury-induced decomposition, contrary to other nitroso species including nitrosamines (RNNOs) and nitrosyl hemes. The samples were homogenized in the previous buffer containing 100 μM DTPA (diethylenetetraminepentaacetic acid) (1:5; FW/V), and centrifuged at $3000 \times g$ for 10 min. Then, the supernatants were incubated with 10 mM NEM (*N*-ethylmaleimide) for 15 min at 4 °C. From each sample, two aliquots were prepared: (a) treated with 10 mM sulfanilamide for 15 min at 4 °C, to eliminate nitrite; and (b) treated with 10 mM sulfanilamide and 7.3 mM $HgCl_2$ for 15 min at 4 °C to eliminate nitrite and *S*-nitrosothiols, respectively. Then, these samples were analyzed in a Nitric Oxide Analyser (NOA 280i, Sievers Instruments, Boulder, CO, USA). The data obtained from the difference between (a) and (b) represented the total RSNOs concentration. The whole procedure was done under red safety light to protect RSNOs from light-dependent decomposition.

2.5. Electrophoretic methods and immunoblot analyses

Polypeptides were separated by 10% SDS–PAGE, and proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon P, Millipore Corp., Bedford, MA) using a Semi-Dry Transfer System (Bio-Rad), as described by Corpas et al. [15]. For immunodetection of nitrotyrosine, a rabbit polyclonal antibody against 3-nitrotyrosine (n-Tyr) [16] diluted 1:3000 was used with an enhanced chemiluminescence kit (ECL-PLUS, Amersham Pharmacia Biotech), and immunoreactive bands were detected with a photographic film (Hyperfilm; Amersham Pharmacia Biotech).

Protein concentration was determined with the Bio-Rad Protein Assay (Hercules, CA), using bovine serum albumin as standard.

2.6. Detection of NO, $O_2^{\cdot-}$ and RSNOs by confocal laser scanning microscopy (CLSM)

NO was detected in olive leaf transversal sections of approximately 25 mm^2 that were incubated for 1 h at 25 °C, in darkness, with 10 μM 4,5-diaminoflorescein diacetate (DAF-2 DA, Calbiochem) prepared in 10 mM Tris–HCl (pH 7.4), according to Corpas et al. [17]. For superoxide radicals, the samples were incubated at 37 °C for 30 min with 10 μM dihydroethidium (DHE, Fluka), as described by Rodríguez-Serrano et al. [18]. After incubation, samples were washed twice in the same buffer for 15 min each. Then, leaf sections were embedded in a mixture of 15% acrylamide–bisacrylamide stock solution as described elsewhere [13], and 80–100 mm-thick sections, as indicated by the vibratome scale, were cut under 10 mM phosphate-buffered saline (PBS). Sections were then soaked in glycerol:PBS (containing azide) (1:1 v/v) and mounted in the same medium for examination with a confocal laser scanning microscope system (Leica TCS SL), using standard filters and collection modalities for DAF-2 green fluorescence (excitation 495 nm; emission 515 nm), DHE green fluorescence (excitation 488 nm; emission 520) and chlorophyll autofluorescence (chlorophyll *a* and *b*, excitation 429 and 450 nm, respectively; emission 650 and 670 nm, respectively) as orange. Background staining, routinely negligible, was controlled with leaf sections unstained or preincubated with the superoxide radical scavenger 2,2,6,6-tetramethyl-piperidine (TMP; 1 mM) for 1 h.

RSNOs were detected using the fluorescent reagent Alexa fluor 488 Hg-link phenylmercury. Olive leaf segments of approximately 25 mm^2 were incubated at 25 °C for 2 h, in darkness, with 10 mM N-ethylmaleimide (NEM) prepared in ethanol, and then were washed three times in 10 mM Tris–HCl buffer, pH 7.4, for 15 min each. The sections were incubated with 10 mM β-mercaptoethanol for 30 min and washed three times with the same buffer for 15 min each. Then, they were incubated with 10 μM Alexa fluor 488 Hg-link phenylmercury (Molecular Probes, cat. no H30462) for 1 h at 25 °C, in darkness. After washing three times in the previous buffer, leaf sections were embedded in a mixture of 15% acrylamide–bisacrylamide stock solution and were processed as described above. The sections were analyzed with a confocal laser scanning microscope system using standard filters for Alexa fluor 488 green fluorescence (excitation 495 nm; emission 519 nm). Three background staining controls were used: (i) sections incubated with NEM plus β-mercaptoethanol and without Alexa fluor 488 Hg-link phenylmercury; (ii) β-mercaptoethanol plus Alexa fluor 488 and without NEM; and (iii) only with β-mercaptoethanol.

2.7. Immunolocalization of nitrotyrosine and GSNO by CLSM

Olive leaves were cut into 4–5 mm pieces and fixed in 4% (w/v) p-formaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 3 h at room temperature. Then they were cryoprotected by immersion in 30% (w/v) sucrose in PB overnight at 4 °C. Serial sections, 60-μm thick, were obtained by means of a cryostat (2800 Frigocut E, Reichert-Jung, Vienna, Austria). The sections were incubated with a rabbit polyclonal antibody against 3-nitrotyrosine [16] diluted 1:300 in TBSA-BSAT, for 3 days at 4 °C. After several washes, sections were incubated with Cy3-labeled anti-rabbit IgG (Amersham) diluted 1:1000 in TBS-BSAT, for 1 h at room temperature, and then mounted in PBS:glycerol 1:1. Controls for background staining, which was

usually negligible, were performed by replacing the corresponding primary antiserum by preimmune serum. For the immunolocalization of GSNO a commercial rat antibody against *S*-nitrosoglutathione (Calbiochem, Cat. no. 487932) diluted 1:2500 was used [19]. Leaf sections were examined with a confocal laser scanning microscope (Leica TCS SL) using standard filters for Cy2-streptavidin (excitation 492 nm; emission, 510 nm) and Cy3-labeled anti-rabbit IgG (excitation 550 nm; emission, 570 nm).

3. Results

3.1. Biochemical parameters of nitrosative stress in olive plants under salinity

The characterization of the enzymatic production of NO from L-arginine (NOS activity) in olive leaf extracts, measured by ozone chemiluminescence, is shown in Fig. 1. The NO production was strictly dependent on the L-Arg concentration, NADPH and calcium. The NOS activity was strongly reduced (90%) in the absence of the cofactors (FAD, FMN and BH_4). Moreover, when the samples were preincubated with the animal NOS inhibitor aminoguanidine (AG) no activity was detected, and when the samples were denatured by heating at 95 °C for 10 min, the NOS activity was reduced 97%.

In a previous study, it was demonstrated that the growth of olive plants with 200 mM NaCl produced a severe reduction in the values of different physiological parameters, including plant growth, photosynthetic rate and chlorophyll content [12]. Using this experimental model, the analysis of the L-arginine-dependent production of NO (NOS activity) showed that salinity produced an increase of 3.6-fold of this activity (Fig. 2A). On the other hand, the level of RSNOs, determined by a chemiluminiscence method, was increased 2-fold by salt stress (Fig. 2B). These data are in agreement with the RSNO detection obtained by CLSM (Fig. 5).

The tyrosine nitration of proteins is considered as an indicator of the peroxynitrite action [20], and the immunoblot analysis of the proteins that could be affected by this process (Fig. 3) showed that NaCl stress caused an increase in the intensity and number of nitrosylated proteins in the molecular mass range between 44 and 60 kDa.

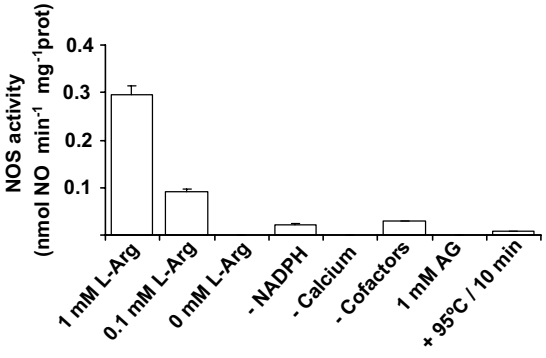

Fig. 1. Characterization of L-arginine-dependent nitric oxide synthase (NOS) activity in olive leaf extracts by ozone chemiluminiscence. Reaction mixtures containing olive leaf extracts were incubated in the absence and presence of L-Arg (1 mM or 0.1 mM), NADPH (1 mM), EGTA (0.5 mM), cofactors (10 μM FAD, 10 μM FMN and 10 μM BH_4), and 1 mM AG (an animal NOS inhibitor). Other reaction mixtures were preincubated at 95 °C for 10 min. Results are means of samples from at least three different experiments.

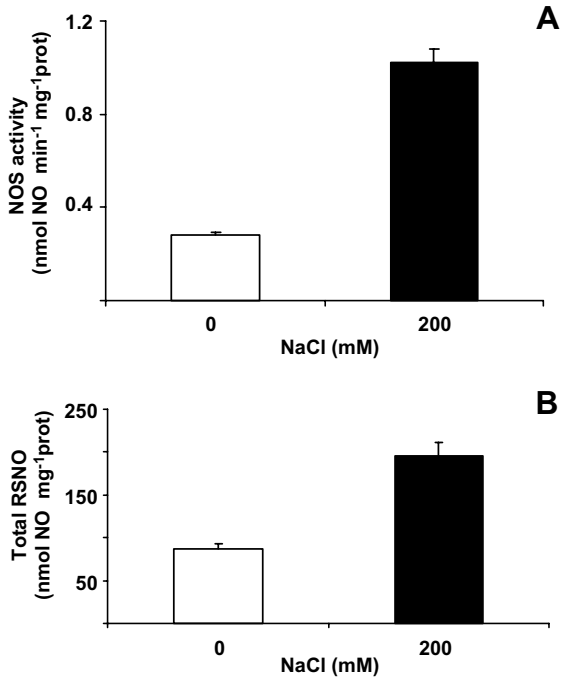

Fig. 2. Analysis of NOS activity and RSNOs by ozone chemiluminiscence in olive leaves. Olive plants were grown without NaCl (control) and with 200 mM NaCl. (A) NO production dependent on L-arginine (NOS activity). (B) Total RSNOs. Results are mean of four different experiments ± S.E.M. Differences from control values were significant at $P < 0.05$.

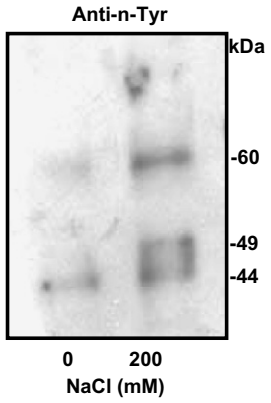

Fig. 3. Protein tyrosine nitration in leaves from olive plants. In leaf extracts from control and NaCl-treated olive plants, 3-nitrotyrosine (n-Tyr) was analyzed by Western blot (30 μg protein per lane) with an antibody against 3-nitrotyrosine (dilution 1/3000).

3.2. Cellular analysis of RNS production induced by salinity

RNS are an important indicator of the presence of NO. The level and cellular localization of nitric oxide (NO), 3-nitrotyrosine (nTyr), GSNO and RSNOs were analyzed in olive leaf sections using either specific antibodies or fluorescent probes (Figs. 4 and 5). In control plants, the localization of endogenous NO by CLSM with DAF-2 DA showed an intense green fluorescence in vascular tissues (xylem and phloem) and a smaller intensity in the upper and lower epidermal cells (Fig. 4A). Under salt stress the green fluorescence was homogenously intensified in all cell types (Fig. 4B).

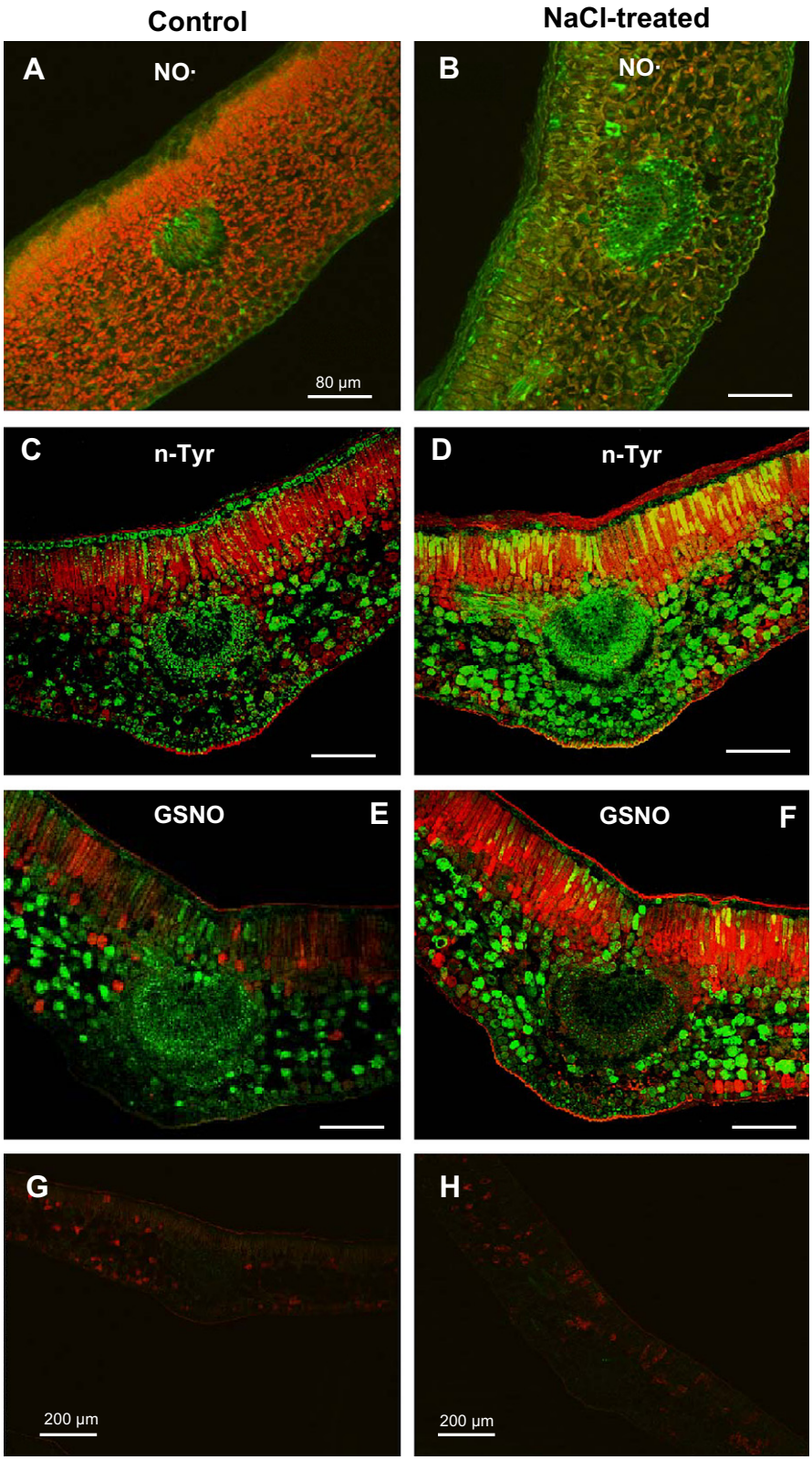

Fig. 4. Representative images illustrating the confocal laser scanning microscopy detection of reactive nitrogen species in olive leaves. Detection of nitric oxide (A and B), nitrotyrosine (n-Tyr; C and D) and *S*-nitrosoglutathione (GSNO; E and F) in olive leaf sections from control (A, C and E) and NaCl-treated plants (B, D and F). NO was detected with 4,5-diaminofluorescein diacetate (DAF-2 DA). Protein 3-nitrotyrosine (n-Tyr) and GSNO were detected using specific antibodies with a dilution of 1:300 and 1:2500, respectively, as described in Section 2. As controls of immunolocalization, primary antibodies to either GSNO and n-Tyr were substituted by pre-immune serum (G) and primary antibodies were omitted (H). The bright green fluorescence corresponded to the detection of NO, n-Tyr and GNSO in the corresponding panels. The orange-yellow color corresponds to the autofluorescence. Each picture was prepared from 30 to 40 cross-sections of olive leaves which were analyzed by CLSM.

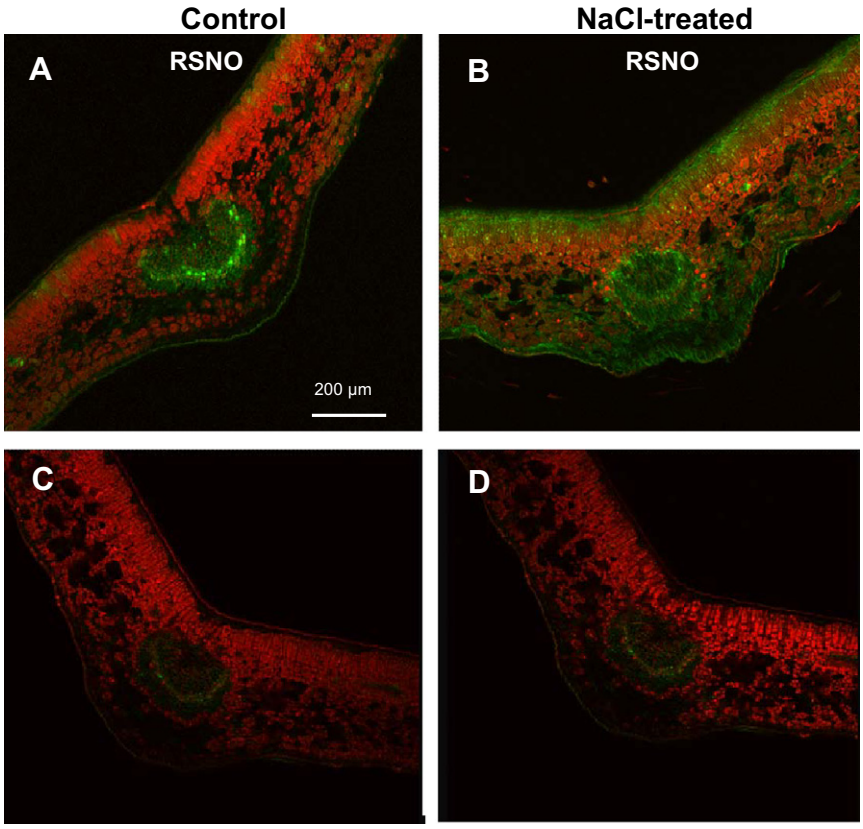

Fig. 5. Representative images illustrating the confocal laser scanning microscopy localization of RSNOs in olive leaves. The detection was carried out using the fluorescent Alexa fluor (AL) 488 Hg-link reagents. (A) Leaf section from control plants, (B) leaf section from NaCl-treated plants, (C) leaf section of control plants incubated without 10 mM *N*-ethyl-maleimide (NEM) and without Alexa Fluor, (D) leaf section of control plants incubated only with NEM. The bright green fluorescence corresponded to RSNOs. The orange-yellow color is due to the chlorophyll autofluorescence. Each picture was prepared from 30 to 40 cross-sections of olive leaves which were analyzed by CLSM.

The tyrosine nitration of proteins was also analyzed using an antibody against nitrotyrosine which recognizes the nitrosylated proteins, as it was shown in Fig. 3. In control leaves, a green fluorescence was observed in vascular tissues, epidermis and spongy mesophyll cells (Fig. 4C). Under salinity, this distribution was very similar but with a significant intensification of the green fluorescence (Fig. 4D). GSNO is thought to function as a natural and mobile reservoir of NO bioactivity [19,21,22]. In control leaves using a specific antibody against GSNO, the green fluorescence was localized in vascular tissues and spongy meshophyll cells (Fig. 4E). In leaves from NaCl-treated plants the distribution of the green fluorescence, attributable to GSNO, was similar to that in control leaves but its intensity was diminished in vascular tissues and was stronger in spongy mesophyll cells (Fig. 4F). In all cases, the orange-yellow color corresponds to the autoflorescence present in all cells. Two controls for background staining were used, where primary antibodies to GSNO and n-Tyr were substituted by pre-immune serum (Fig 4G and H). Under these conditions, no immunofluorescence background was observed.

RSNOs were detected using a new commercial fluorescent probe, Alexa Fluor 488 Hg-link (AF) that can react with nitrosylated thiols (RSNOs) via the Saville reaction. In control leaves, the green fluorescence attributable to RSNOs was present mainly in phloem (Fig. 5A). In leaves from NaCl-treated plants, the distribution of RSNOs was diminished in vascular tissue but was stronger in spongy mesophyll and epidermal cells (Fig. 5B). These results were parallel to those obtained in the immunolocalization of GSNO (Fig. 4E and F). When *N*-ethyl maleimide (NEM) and Alexa Fluor 488 Hg-link were omitted in the incubation mixture, the green fluorescence was almost undetectable (Fig. 5C), and similar results were obtained when AF was added and NEM omitted (Fig. 5D). In all cases, the red color corresponds to the autofluorescence distributed in almost all leaf cells.

3.3. Cellular analysis of superoxide radical production induced by salinity

The reaction of $NO^{\cdot}$ with the superoxide radical ($O_2^{\cdot-}$) generates peroxynitrite which has been shown to mediate the tyrosine nitration of proteins. To get deeper insights into this process, the cellular production of $O_2^{\cdot-}$ was analyzed by CLSM using the fluorescent probe DHE, which is specific for these radicals [23]. In control leaves, the green fluorescence corresponding to $O_2^{\cdot-}$ was present in vascular tissues and epidermic cells (Fig. 6A), a similar localization to that of NO (Fig. 4A). However, in leaves from NaCl-treated plants, the green fluorescence was intensified, with a homogenous distribution in all cell types (vascular tissue, epidermic cells, and spongy and palisade mesophyll) (Fig. 6B). When leaf samples from NaCl-treated plants were preincubated with 1 mM TMP (a superoxide radical scavenger), the green fluorescence was considerably reduced (Fig. 6C), indicating the specificity of the DHE probe for superoxide radicals. The appearance of an

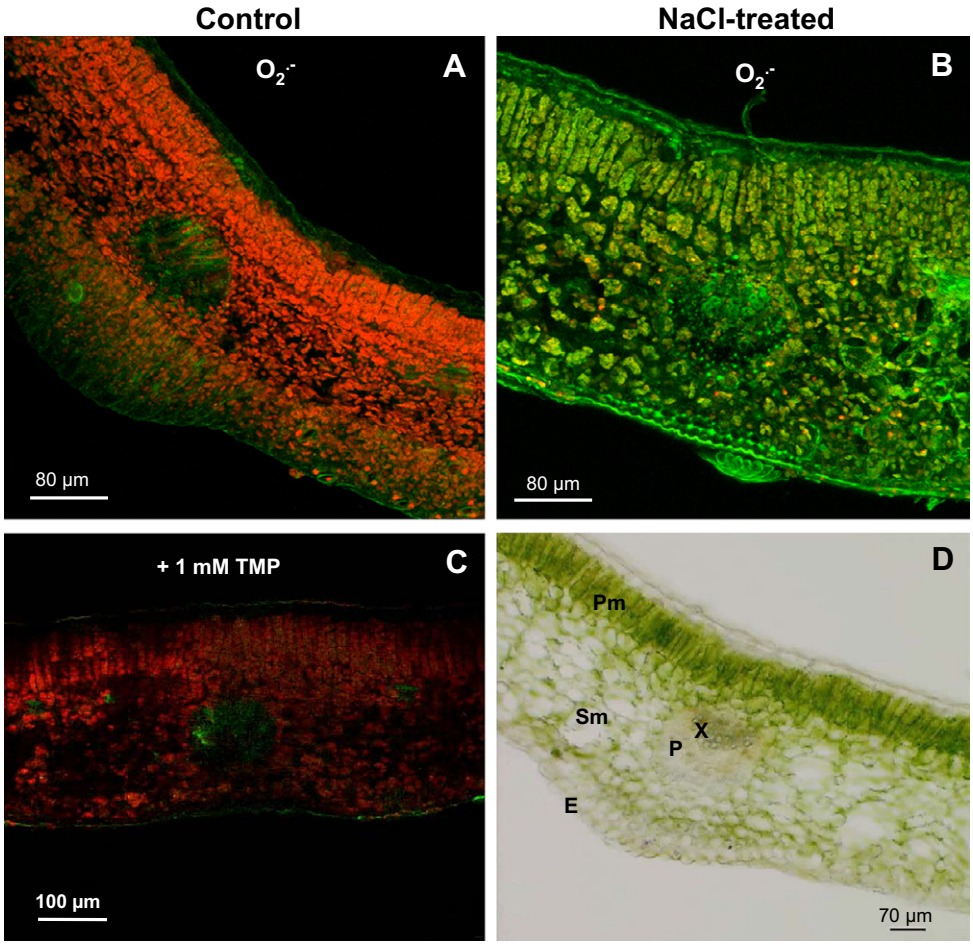

Fig. 6. Representative images illustrating the confocal laser scanning microscopy detection of superoxide radicals in olive leaves. Superoxide radicals ($O_2^{\cdot-}$) were detected in olive leaf sections with dihydroethidium (DHE) by the bright green fluorescence produced. (A) leaf section from control plants. (B) leaf section from NaCl-treated plants. (C) leaf section from NaCl-treated plants preincubated with the superoxide radical scavenger TMP (1 mM) (negative control). (D) bright-field image of an olive leaf section from control plants without any treatment, showing its different tissues. The orange-yellow color corresponds to the chlorophyll autofluorescence. Each picture was prepared from 30 to 40 leaf cross-sections which were analyzed by CLSM. E, epidermis; P, phloem; Pm, palisade mesophyll; Sm, spongy mesophyll; X, xylem. Scale bar represents 80 µm (A–C) and 70 µm (D).

olive leaf section under the optical microscope showing its different tissues is presented in Fig. 6D. In all cases, the orange-yellow color corresponds to the autofluorescence distributed in almost all leaf cells.

4. Discussion

In animal cells, nitric oxide and other RNS, such as peroxynitrite and GSNO, have attracted the attention of many researchers due to the physiological repercussions of their metabolism and because these species can also cause extensive cellular damage if are overproduced without control [20,24]. However, in plants there is very little information on these NO-related molecules. In this work, the modulation of reactive nitrogen species (NO, GSNO and total RSNOs) was studied in olive plants under salinity and 3-nitrotyrosine was used as a marker of nitrosative stress with the purpose of establishing a correlation between abiotic and nitrosative stress.

In plants there are very few reports on the specific activity of L-arginine-dependent nitric oxide synthases [10] perhaps due to the difficulties in measuring this activity. In our work, the NOS activity in olive leaves was measured by an ozone chemiluminiscence method, set up for plant tissues, which has been demonstrated to be very sensitive [10]. In olive leaves the NOS activity determined was 0.280 nmol NO mg^{-1} protein min^{-1}. This activity was inhibited by aminoguanidine a well-known inhibitor of inducible and constitutive NOSs in animal cells [13], it was dependent on L-arginine and required NADPH, calcium and different cofactors (FAD, FMN and BH_4). However, it must be mentioned that to our knowledge there are no reports on the presence of BH_4 in plants. This cofactor promotes and/or stabilizes the active dimeric form of the three mammal NOS isoforms [25]. In plants, this function perhaps could be carried out by tetrahydrofolate (FH_4) whose biosynthesis and distribution is well known in higher plants [26]. However, further research is necessary to demonstrate this function of FH_4 as a NOS cofactor in plants. On the other hand, the activity of the commercial rat neuronal NOS, used as positive control in this work, was 200 nmol NO mg^{-1} protein min^{-1}. The NOS activity of olive leaves (ca. 0.300 nmol NO mg^{-1} protein min^{-1}) was similar to that previously reported in pea leaf extracts (0.120 nmol NO mg^{-1} protein min^{-1}) [10] and sunflower hypocotyl extracts

(0.294 nmol NO mg^{-1} protein min^{-1}) (Chaki et al., unpublished results) and *Lupinus albus* nodules (0.296 nmol citrulline mg^{-1} protein min^{-1}) [27]. These specific activity values are 50–100 times higher than those previously reported for the NOS activity in *Mucuna hassjoo* [28], TMV-infected tobacco leaves [29], and roots and leaves of *Zea mays* [30].

In plants, apart from the NOS-like activity, other documented sources of NO are two nitrate reductases [31], different plant organelles like peroxisomes [17], mitochondria [31] and chloroplasts [32], and also non-enzymatic systems [6,33]. The AtNOS1 protein which until very recently was considered as the characteristic plant NOS [34] has just been demonstrated that does not produce NO and, therefore, is not a real L-arginine-dependent NOS enzyme [35,36]. The NOS activity values reported in this work cannot be easily compared with the values of NO emitted by certain plants [37,38], because the NOS activity determined must be just a part of the whole endogenous production of NO.

A pharmacological study has shown that NO can function as a signal of salt resistance in calluses from reed plants [39]. In olive leaves, the accumulation of NO evaluated by NOS activity (Fig. 2A) and CLSM with DAF-2 DA (Fig. 4B) was increased by salt stress. A similar pattern was observed for the accumulation of superoxide radicals under salt stress conditions (Fig. 6B). It is well established that superoxide radicals ($O_2^{\cdot-}$) and NO can react to form peroxynitrite [20], a very powerful oxidant which can react directly with cysteine, methionine and tryptophan residues [40]. With the discovery that peroxynitrite is generated in vivo and the demonstration that in animal cells peroxynitrite can modify a number of biological molecules, including proteins, lipids, and nucleic acids [40,41], a considerable attention has been given to the role of peroxynitrite in cellular oxidative damage. One of the changes produced by peroxynitrite is the nitration of tyrosine residues in the ortho position, which are used as biomarkers of nitrosative stress in diseases [24,41].

In plants, to our knowledge, there is no information on the effect of salinity on the tyrosine nitration of proteins. Using an antibody against n-Tyr, the results obtained in this work indicated that in olive leaves salt stress produced an increase in the number and intensity of proteins of 44–60 kDa that experimented tyrosine nitration (Fig. 3), and also a general intensification of the tyrosine-nitrated proteins in all cells (Fig. 4D). This cellular localization is coincident with those of NO (Fig. 4B) and superoxide radicals (Fig. 6B). In nitrite reductase antisense tobacco leaves the induction of several tyrosine-nitrated polypeptides with molecular masses between 10-kDa and 50-kDa was described [42]. Moreover, in tobacco BY-2 suspension cells treated with a fungal elicitin, the induction of tyrosine nitration in proteins with molecular masses in the range 20–50 kDa was demonstrated [43]. Conversely, in tobacco transgenic plants with genetically increased cytokinin levels the content of tyrosine nitrated proteins decreased [44].

In animals a proteomic study using a monoclonal antibody against nitrotyrosine allowed the identification of over 40 different proteins during inflammatory challenge in rat lung and liver, which were involved in different functions such as oxidative stress, apoptosis, ATP production, and fatty acid metabolism [45]. Recently, in *Arabidopsis* plants treated with gaseous NO until 52 proteins were identified in leaves that represented candidates for another type of post-translational modification, *S*-nitrosylation [46]. These included stress-related, redox-related, signalling/regulating, cytoskeleton, and metabolic proteins [46]. All these data indicate that NO and NO-derived molecules have a relevant physiological role in plants under normal and stress conditions. In this work, we have also observed that salinity causes an induction of *S*-nitrosothiols (Figs. 2B and Fig. 5B).

At cellular level, analysis of NO, 3-nitrotyrosine, RSNO and GSNO revealed that salinity caused a general rise of these RNS in all cell types of olive leaves (Figs. 4 and 5). In control plants NO was mainly localized in vascular tissues, which is in agreement with previous data reported in pea plants [10,17], but salinity produced a general increase of NO in palisade and spongy mesophyll cells besides vascular cells. This result is consistent with the enzymatic production of NO from L-arginine (NOS activity) observed in olive leaf extracts from salt-stressed plants. These data also agree with results reported in leaf cells of tobacco plants, where a rapid and significant increase of NO, evaluated by fluorescence, was induced by heat, osmotic and salt stresses [47]. In contrast, natural senescence and cadmium stress produced in pea plants a decrease of the NO production in leaves [17,19].

In plant tissues there is very little information on the cellular localization of GSNO and *S*-nitrosylated proteins. To our knowledge, the only evidence available is the recent demonstration of the presence of GSNO in collenchyma cells of pea plants [19]. Thus, the detection of RSNO and GSNO mainly in spongy mesophyll and vascular tissues of olive leaves, and the sensitivity of this RNS to salt stress suggest that the redistribution of GSNO-derived NO from vascular tissues can have an important function in nitrosative stress and probably in signalling-related processes.

In conclusion, the results presented in this work indicate that in leaves from olive plants salt stress produces an increase in the RNS NO, GSNO and RSNO, and as a result of this, there is a rise in tyrosine-nitrated proteins, which are considered biomarkers of nitrosative stress. These data indicate that in olive leaves salinity not only induces oxidative stress, as has been previously reported [12], interestingly, this oxidative situation is also accompanied by nitrosative stress. However, this does not mean that oxidative and nitrosative stress necessarily overlap in all abiotic toxic conditions. Cadmium is known to induce oxidative stress in pea plants [48,49] but recently it was reported that Cd does not cause nitrosative stress in these plants [19]. The results described in this work apart from providing new insights into the physiological response of olive plants to NaCl stress, also evidence that vascular tissues could play in plants an important function in the redistribution of RNS (NO and/or RSNOs) during normal and stress conditions. These considerations perhaps could be extended to other plant species under different abiotic stress situations.

Acknowledgments: Olive seeds were kindly provided by the *Dpto. de Olivicultura y Arboricultura Frutal, Banco de Germoplasma Mundial*, CIFA, Córdoba, Spain. The valuable help of Prof. Fernando Pliego and Dr. Araceli Barceló (CIFA of Churriana, Málaga, Spain) in setting up the in vitro tissue culture conditions of olive plants is appreciated. We specially acknowledge Prof. José Rodrigo, Instituto de Neurobiología Ramón y Cajal, CSIC, Madrid, for his generous donation of the antibody against 3-nitrotyrosine. Special thanks are given to Miss Nieves de la Casa-Adán (Technical Services of the University of Jaén) for her technical assistance in the confocal laser scanning microscopy analyses and Miss Emperatriz Córdoba for the maintenance of *in vitro* plant cultures. This work was supported by grants

from the Ministry of Education and Science (BIO2006-14949-C02-01, BIO2006-14949-C02-02) and *Junta de Andalucía* (Groups CVI 286 and CVI 192).

References

[1] Hausladen, A. and Stamler, J.S. (1999) Nitrosative stress. Methods Enzymol. 300, 389–395.
[2] Halliwell, B. and Gutteridge, J.M. (2000) Free Radic. Biol. Med. (Oxford, UK).
[3] Klatt, P. and Lamas, S. (2000) Regulation of protein function by *S*-glutathiolation in response to oxidative and nitrosative stress. Eur. J. Biochem. 267, 4928–4944.
[4] Neill, S.J., Desikan, R., Clarke, A. and Hancock, J.T. (2003) Nitric oxide signalling in plants. New Phytol. 159, 11–35.
[5] Lamattina, L., García-Mata, C., Graziano, M. and Pagnussat, G. (2003) Nitric oxide: the versatility of an extensive signal molecule. Annu. Rev. Plant Biol. 54, 109–136.
[6] del Río, L.A., Corpas, F.J. and Barroso, J.B. (2004) Nitric oxide and nitric oxide synthase activity in plants. Phytochemistry 65, 783–792.
[7] Shapiro, A.D. (2005) Nitric oxide signaling in plants. Vitam. Horm. 72, 339–398.
[8] Yamasaki, H. (2005) Nitric oxide in plant biology: its past and the future in: Nitric Oxide Signaling in Higher Plants (Magalhaes, P., Rana, P., Singh, R.N. and Passos, L.P., Eds.), ISBN 0-9761849-2-3, pp. 1–23, Studium Press, LLC, Houston, USA.
[9] Delledonne, M. (2005) NO news is good news for plants. Curr. Opin. Plant Biol. 8, 390–396.
[10] Corpas, F.J., Barroso, J.B., Carreras, A., Valderrama, R., Palma, J.M., León, A.M., Sandalio, L.M. and del Río, L.A. (2006) Constitutive arginine-dependent nitric oxide synthase activity in different organs of pea seedlings during plant development. Planta 224, 246–254.
[11] Hasegawa, P.M., Bressan, R.A., Zhu, J.K. and Bohnert, H.J. (2000) Plant cellular and molecular responses to high salinity. Annu. Rev. Plant Physiol. Plant Mol. Biol. 51, 463–499.
[12] Valderrama, R., Corpas, F.J., Carreras, R., Gómez-Rodríguez, M.V., Chaki, M., Pedrajas, J.R., Fernández-Ocaña, A., del Río, L.A. and Barroso, J.B. (2006) The dehydrogenase-mediated recycling of NADPH is a key antioxidant system against salt-induced oxidative stress in olive plants. Plant Cell Environ. 29, 1449–1459.
[13] Laszlo, F., Evans, S.M. and Whittle, B.J. (1995) Aminoguanidine inhibits both constitutive and inducible nitric oxide synthase isoforms in rat intestinal microvasculature in vivo. Eur. J. Pharmacol. 272, 169–175.
[14] Jourd'Heuil, D., Jourd'heuil, F.L., Lowery, A.M., Hughes, J. and Grisham, M.B. (2005) Detection of nitrosothiols and other nitroso species in vitro and in cells. Methods Enzymol. 396, 118–131.
[15] Corpas, F.J., Barroso, J.B., Sandalio, L.M., Distefano, S., Palma, J.M., Lupiáñez, J.A. and del Río, L.A. (1998) A dehydrogenase-mediated recycling system of NADPH in plant peroxisomes. Biochem. J. 330, 777–784.
[16] Uttenthal, L.O., Alonso, D., Fernández, A.P., Campbell, R.O., Moro, M.A., Leza, J.C., Lizasoain, I., Esteban, F.J., Barroso, J.B., Valderrama, R., Pedrosa, J.A., Peinado, M.A., Serrano, J., Richart, A., Bentura, M.L., Santacana, M., Martinez-Murillo, R. and Rodrigo, J. (1998) Neuronal and inducible nitric oxide synthase and nitrotyrosine immunoreactivities in the cerebral cortex of the aging rat. Microsc. Res. Tech. 43, 75–88.
[17] Corpas, F.J., Barroso, J.B., Carreras, A., Quirós, M., León, A.M., Romero-Puertas, M.C., Esteban, F.J., Valderrama, R., Palma, J.M., Sandalio, L.M., Gómez, M. and del Río, L.A. (2004) Cellular and subcellular localization of endogenous nitric oxide in young and senescent pea plants. Plant Physiol. 136, 2722–2733.
[18] Rodríguez-Serrano, M., Romero-Puertas, M.C., Zabalza, A., Corpas, F.J., Gómez, M., del Río, L.A. and Sandalio, L.M. (2006) Cadmium effect on oxidative metabolism of pea (*Pisum sativum* L.) roots. Imaging of reactive oxygen species and nitric oxide accumulation in vivo. Plant Cell Environ. 29, 1532–1544.
[19] Barroso, J.B., Corpas, F.J., Carreras, A., Rodríguez-Serrano, M., Esteban, F.J., Fernández-Ocaña, A., Chaki, M., Romero-Puertas, M.C., Valderrama, R., Sandalio, L.M. and del Río, L.A. (2006) Localization of *S*-nitrosoglutathione and expression of *S*-nitrosoglutathione reductase in pea plants under cadmium stress. J. Exp. Bot. 57, 1785–1793.
[20] Radi, R. (2004) Nitric oxide, oxidants, and protein tyrosine nitration. Proc. Natl. Acad. Sci. USA 101, 4003–4008.
[21] Durner, J., Gow, A.J., Stamler, J.S. and Glazebrook, J. (1999) Ancient origins of nitric oxide signaling in biological systems. Proc. Natl. Acad. Sci. USA 96, 14206–14207.
[22] Díaz, M., Achkor, H., Titarenko, E. and Martínez, M.C. (2003) The gene encoding glutathione-dependent formaldehyde dehydrogenase/GSNO reductase is responsive to wounding, jasmonic acid and salicylic acid. FEBS Lett. 543, 136–139.
[23] Zhao, H., Kalivendi, S., Zhang, H., Joseph, J., Nithipatikom, K., Vázquez-Vivar, J. and Kalyanaraman, B. (2003) Superoxide reacts with hydroethidine but forms a fluorescent product that is distinctly different from ethidium: potential implications in intracellular fluorescence detection of superoxide. Free Radic. Biol. Med. 34, 1359–1368.
[24] Ischiropoulos, H. (1998) Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species. Arch. Biochem. Biophys. 356, 1–11.
[25] Alderton, W.K., Cooper, C.E. and Knowles, R.G. (2001) Nitric oxide synthases: structure, function and inhibition. Biochem. J. 357, 593–615.
[26] Sahr, T., Ravanel, S. and Rebeille, F. (2005) Tetrahydrofolate biosynthesis and distribution in higher plants. Biochem. Soc. Trans. 33, 758–762.
[27] Cueto, M., Hernández-Perera, O., Martin, R., Bentura, M.L., Rodrígo, J., Lamas, S. and Golvano, M.P. (1996) Presence of nitric oxide synthase activity in roots and nodules of *Lupinus albus*. FEBS Lett. 398, 159–164.
[28] Ninnemann, H. and Maier, J. (1996) Indications for the occurrence of nitric oxide synthases in fungi and plants and the involvement in photocondition of *Neurospora crassa*. Photochem. Photobiol. 64, 393–398.
[29] Durner, J., Wendehenne, D. and Klessig, D.F. (1998) Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. Proc. Natl. Acad. Sci. USA 95, 10328–10333.
[30] Ribeiro Jr., E.A., Cunha, F.Q., Tamashiro, W.M. and Martins, I.S. (1999) Growth phase-dependent subcellular localization of nitric oxide synthase in maize cells. FEBS Lett. 445, 283–286.
[31] Planchet, E. and Kaiser, W.M. (2006) Nitric oxide production in plants: facts and fictions. Plant Signaling Behavior 1, 46–51.
[32] Jasid, S., Simontacchi, M., Bartoli, C.G. and Puntarulo, S. (2006) Chloroplasts as a nitric oxide cellular source. effect of reactive nitrogen species on chloroplastic lipids and proteins. Plant Physiol. 142, 1246–1255.
[33] Bethke, P.C., Badger, M.R. and Jones, R.L. (2004) Apoplastic synthesis of nitric oxide by plant tissues. Plant Cell. 16, 332–341.
[34] Guo, F.Q., Okamoto, M. and Crawford, N.M. (2003) Identification of a plant nitric oxide synthase gene involved in hormonal signaling. Science 302, 100–103.
[35] Zemojtel, T., Fröhlich, A., Palmieri, M.C., Kolanczyk, M., Mikula, I., Wyrwicz, L.S., Wanker, E.E., Mundlos, S., Vingron, M., Martasek, P. and Durner, J. (2006) Plant nitric oxide synthase: a never-ending story? Trends Plant Sci. 11, 524–525.
[36] Crawford, N.M., Galli, M., Tischner, R., Heimer, Y.M., Okamoto, M. and Mack, A. (2006) Response to Zemojtel et al: plant nitric oxide synthase: back to square one. Trends Plant Sci. 11, 526–527.
[37] Leshem, Y.Y. and Haramaty, E. (1996) The characterization and contrasting effects of the nitric oxide free radical in vegetative stress and senescence of *Pisum sativum* Linn. foliage. J. Plant. Physiol. 148, 258–263.
[38] Planchet, E., Jagadis Gupta, K., Sonoda, M. and Kaiser, W.M. (2005) Nitric oxide emission from tobacco leaves and cell suspensions: rate limiting factors and evidence for the involvement of mitochondrial electron transport. Plant J. 41, 732–743.
[39] Zhao, L., Zhang, F., Guo, J., Yang, Y., Li, B. and Zhang, L. (2004) Nitric oxide functions as a signal in salt resistance in the calluses from two ecotypes of reed. Plant Physiol. 134, 849–857.

[40] Álvarez, B. and Radi, R. (2003) Peroxynitrite reactivity with amino acids and proteins. Amino Acids 25, 295–311.
[41] Dalle-Donne, I., Scaloni, A., Giustarini, D., Cavarra, E., Tell, G., Lungarella, G., Colombo, R., Rossi, R. and Milzani, A. (2005) Proteins as biomarkers of oxidative/nitrosative stress in diseases: the contribution of redox proteomics. Mass Spectrom. Rev. 24, 55–99.
[42] Morot-Gaudry-Talarmain, Y., Rockel, P., Moureaux, T., Quillere, I., Leydecker, M.T., Kaiser, W.M. and Morot-Gaudry, J.F. (2002) Nitrite accumulation and nitric oxide emission in relation to cellular signaling in nitrite reductase antisense tobacco. Planta 215, 708–715.
[43] Saito, S., Yamamoto-Katou, A., Yoshioka, H., Doke, N. and Kawakita, K. (2006) Peroxynitrite generation and tyrosine nitration in defense responses in tobacco BY-2 cells. Plant Cell Physiol. 47, 689–697.
[44] Wilhelmova, N., Fuksova, H., Srbova, M., Mikova, D., Mytinova, Z., Prochazkova, D., Vytasek, R. and Wilhelm, J. (2006) The effect of plant cytokinin hormones on the production of ethylene, nitric oxide, and protein nitrotyrosine in ageing tobacco leaves. Biofactors 27, 203–211.
[45] Aulak, K.S., Miyagi, M., Yan, L., West, K.A., Massillon, D., Crabb, J.W. and Stuehr, D.J. (2001) Proteomic method identifies proteins nitrated in vivo during inflammatory challenge. Proc. Natl. Acad. Sci. USA 98, 12056–12061.
[46] Lindermayr, C., Saalbach, G. and Durner, J. (2005) Proteomic identification of *S*-nitrosylated proteins in *Arabidopsis*. Plant Physiol. 137, 921–930.
[47] Gould, K.S., Lamotte, O., Klinguer, A., Pugin, A. and Wendehenne, D. (2003) Nitric oxide production in tobacco leaf cells: a generalized stress response? Plant Cell Environ. 26, 1851–1862.
[48] Romero-Puertas, M.C., Palma, J.M., Gómez, M., del Río, L.A. and Sandalio, L.M. (2002) Cadmium causes the oxidative modification of proteins in pea plants. Plant Cell Environ. 25, 677–686.
[49] Romero-Puertas, M.C., Rodríguez-Serrano, M., Corpas, F.J., Gómez, M., del Río, L.A. and Sandalio, L.M. (2004) Cadmium-induced subcellular accumulation of $O_2^{\cdot-}$ and H_2O_2 in pea leaves. Plant Cell Environ. 27, 1122–1134.